Two ATP-binding cassette transporters involved in (S)-2-aminoethyl-cysteine uptake in *Thermus thermophilus*

**Running title:** AEC transporters of *T. thermophilus*

Yuko Kanemaru*, Fumihito Hasebe*, Takeo Tomita, Tomohisa Kuzuyama, and Makoto Nishiyama

1Biotechnology Research Center, The University of Tokyo, Tokyo 113-8657, Japan
2RIKEN SPring-8 Center, Hyogo 679-5148, Japan

**Corresponding author:** Makoto Nishiyama, Ph.D.

Biotechnology Research Center, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan

Fax: +81-3-5841-8030; Phone: +81-3-5840-3074

E-mail: umanis@mail.ecc.u-tokyo.ac.jp

*Those authors contributed equally
ABSTRACT

Thermus thermophilus exhibits hypersensitivity to a lysine analog, (S)-2-aminoethyl-cysteine (AEC). Cosmid libraries were constructed using genomes from two AEC-resistant mutants, AT10 and AT14, and the cosmids that conferred AEC resistance on the wild-type strain were isolated. When the cosmid library for mutant AT14 was screened, two independent cosmids conferring AEC resistance to the wild type were obtained. Two cosmids carried a common genomic region from TTC0795 to TTC0810. This region contains genes encoding two different ATP-binding cassette (ABC) transporters: one consisting of TTC0806/TTC0795 and the other consisting of TTC0967/TTC0968/TTC0969/TTC0970 using TTC0807 and TTC0966 as the periplasmic substrate-binding protein, respectively. Sequencing revealed that AT14 carries mutations in TTC0795 and TTC0969, causing decreases in the thermostability of the products. By similar screening for cosmids constructed for the mutant AT10, mutations were found at TTC0807 and TTC0969. Mutation in either of the transporter components gave partial resistance to AEC in the wild-type strain, while mutations of both transporters conferred complete AEC resistance. This result indicates that both transporters are involved in AEC uptake in T. thermophilus. To elucidate the mechanism of AEC uptake, crystal structures of TTC0807 were determined in several substrate-binding forms. The structures revealed that TTC0807 recognizes various basic amino acids by changing the side-chain conformation of Glu19, which interacts with the side-chain amino groups of the substrates.
Resistance to the structural analogues of amino acids can be due to different mechanisms in cells, such as increased degradation or reduced uptake of the analogue, and decreased sensitivity of the target enzyme. (S)-2-aminoethyl-cysteine (AEC, Fig. 1) is known to be a lysine analogue. In *Escherichia coli* and *Bacillus subtilis*, lysine-binding L-box, a regulatory leader sequence, is present upstream of the corresponding lysine biosynthetic genes and mutations in L-box provide resistance to AEC by increasing the production of aspartate kinase (AK) (1, 2), which catalyzes the first step in the lysine biosynthetic pathway. In addition to the regulatory leader regions, mutations are found in the regulatory domain of AK, which causes decreased sensitivity to AEC-mediated feedback inhibition (3). In both cases, mutations widen the flow toward lysine biosynthesis to cause the accumulation of a large amount of lysine, which in turn titrates out AEC to avoid its mis-incorporation into protein. As a different target of AEC, lysyl-tRNA synthetase is proposed based on the isolation of mutants that contain lysyl-tRNA synthetases with lower affinity to AEC (4).

An extremely thermophilic bacterium, *Thermus thermophilus*, exhibits hypersensitivity to AEC. The growth of *T. thermophilus* was inhibited by AEC at 10 µM, which contrasts with other bacteria, such as *Escherichia coli* and *C. glutamicum*, which grow even in the presence of 5,000 µM AEC. In most bacteria, lysine is synthesized through the diaminopimelate (DAP) pathway; however, it is synthesized through the α-aminoadipate (AAA) pathway in *T. thermophilus* (5, 6). In *T. thermophilus*, in addition to the repression by lysine of the expression of genes involved in lysine biosynthesis (7), lysine biosynthesis is regulated through feedback inhibition by lysine of homocitrate synthase (HCS), which catalyzes the first step in lysine biosynthesis (8). Lysine at µM levels inhibits HCS of *T. thermophilus* (8). As in the above instances, it is possible that the growth inhibition of *T. thermophilus* by AEC might be due to the inhibition of HCS; however, we also found that the growth of a *T. thermophilus* mutant possessing HCS with H72L replacement, which is feedback-resistant to high concentrations of lysine (9), was...
inhibited by AEC with the same sensitivity as that of the wild-type strain. This observation suggests that AEC acts on a target other than HCS to inhibit the growth of *T. thermophilus*. In this study, we isolated AEC-resistant mutants of *T. thermophilus* and tried to elucidate the mechanism of the hypersensitivity to AEC of *T. thermophilus* by identification of the mutated genes, and found that there are two different systems to transport AEC into cells. To elucidate the mechanism of AEC uptake in detail, crystal structures of a periplasmic substrate-binding protein were determined.
MATERIALS AND METHODS

Strains, media, and chemicals. E. coli DH5α (10) was used for DNA manipulation, and E. coli BL21-Codon-Plus (DE3)-RIL [F-, ompT, hsdS (r_{HI}, m_{HI}), dcm^+, Tel', gal, (DE3), endA, Hhe, [argU, ileY, leuW, Cam']] (Stratagene, La Jolla, CA) was used as the host to express TTC0795, TTC0806, TTC0807, and TTC0969 genes. The 2 x YT medium (10) was generally used for cultivation of E. coli cells, whereas TM medium (nutrient medium) (11) and MM medium (minimal medium) (12), were used for cultivation of T. thermophilus HB27 and mutant strains. Antibiotics and isopropyl β-D-thiogalactopyranoside (IPTG) were added to the medium when required. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO), Wako Pure Chemical (Osaka, Japan), and Kanto Chemicals (Tokyo, Japan). Enzymes for DNA manipulation were purchased from Takara Shuzo (Kyoto, Japan) and TOYOBO (Osaka, Japan).

Chemical mutagenesis of T. thermophilus and screening of AEC-resistant strain. To identify the genes responsible for AEC hypersensitivity, we isolated AEC-resistant strains of T. thermophilus by the following procedures. T. thermophilus cells cultured in TM (72 ml) were washed and suspended in 48 ml buffer I (500 mM Tris-HCl, pH8.0, 1 mg ml\(^{-1}\) N-methyl-N'-nitro-N-nitrosoguanidine (NTG)). Suspended cells were cultured at 70 °C for 60 min without shaking. The cells were harvested by centrifugation, 10,000 x g, for 10 min at 4 °C, washed with sterile water, and suspended in 20 ml MM medium. The cells were spread on an MM medium gellan gum plate containing 500 μM AEC. After 4 days’ cultivation at 70 °C, eight first-growing colonies were isolated as AEC-resistant mutants.

Isolation of DNA fragments responsible for AEC resistance. Genomic DNAs from AEC-resistant mutants were purified and partially digested with Sau3AI. DNA
fragments larger than 20 kb were ligated to BamHI- and phosphatase-treated pOJ446 cosmid vector (13), packaged with a LAMBDa INN packaging kit (Nippon-Gene, Tokyo, Japan), and introduced into *E. coli* XL1-Blue MRF’ cells according to the manufacturer’s instructions. For every AEC mutant, about 200 colonies were obtained, sufficient to cover the whole genome of *T. thermophilus*. Cosmid DNA was purified from each *E. coli* colony and used as the cosmid library, which was pooled to transform *T. thermophilus* HB27 (11). *T. thermophilus* HB27 cells transformed with the cosmid library were grown in liquid MM medium supplemented with 500 μM AEC at 70 °C. Cosmids that gave AEC resistance to the wild-type strain were selected as candidates that carry mutations responsible for showing AEC resistance.

**Thermostability of mutated ABC transporter components.** All PCR primers used are listed in Supplemental Table S1. TTC0795 and TTC0969 of mutant AT14 were prepared as follows. TTC0795 was prepared in a form with a Strep-tag at the N-terminus in *E. coli* BL21 RIL-Codon Plus (DE3) cells using pET26b (+) as the expression vector. Harvested cells were suspended in 8 ml buffer II (20 mM Tris-HCl, pH8.0, 150 mM NaCl), washed, and disrupted by sonication. The supernatant prepared by centrifugation at 40,000 x g for 20 min was applied to a Strep-tactin column pre-equilibrated with buffer III (100 mM Tris-HCl, pH8.0, 150 mM NaCl). After washing with the same buffer, adsorbed proteins were eluted with buffer III supplemented with 2.5 mM desthiobiotin. TTC0969 with a His8-tag was purified using an Ni²⁺ affinity column. Buffer II supplemented with 20 mM or 500 mM imidazole was used for column pre-equilibration and protein elution, respectively. Protein concentrations were determined by the Bradford method using a Bio-Rad protein assay kit (Bio-Rad, Tokyo). After the protein concentration had been adjusted to 1 mg ml⁻¹, protein samples were heated at appropriate temperatures for 30 min. These samples were then cooled on ice for 10 min and centrifuged at 20,000 x g for 10 min to remove aggregates. The supernatants were applied...
to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

**Preparation of knockout mutants of** *T. thermophilus*. To verify the involvement of the mutation found in the isolated cosmid in AEC resistance, *T. thermophilus* mutants were constructed, each lacking *TTC0807*, *TTC0969*, or both *TTC0807/TTC0969*. The plasmid to delete the *TTC0807* gene was constructed as follows. The first PCR was performed with primers ttc0807-up-fw and ttc0807-up-rv to amplify the upstream region of the *TTC0807* gene of 700 bp. The second PCR was performed with primers htk-fw and htk-rv to amplify the *HTK* (hyper-thermostable kanamycin nucleotidyltransferase) gene (14). The third PCR was performed with primers ttc0807-down-fw and ttc0807-down-rv to amplify the downstream region (700 bp) of the *TTC0807* gene. Each of the three amplified fragments was digested with XbaI/SpeI, SpeI/BamHI, and BamHI/XhoI, respectively, and cloned separately into pBlueScript II KS(+) for sequence verification. Three fragments with the correct sequences were ligated together with pBlueScript II KS(+) digested with XbaI/XhoI. The resulting plasmid, named pKS-d0807, was used to knockout *TTC0807* of *T. thermophilus* HB27. Colonies that were resistant to 200 μg/ml kanamycin on TM medium were picked up, and the knockout was confirmed by PCR using ttc0807 check-fw and ttc0807 check-rv. Other genes (*TTC0969* and *TTC0807*) were knocked out in a similar way. In some cases, the *hyg10* gene encoding the thermostable hygromycin resistance gene (15) was used in place of the *HTK* gene. All PCR primers used for gene disruption are listed in Supplemental Table S2. Complete cloning methods (thermo cycling conditions, polymerase enzymes used etc.) are available from the corresponding author upon request.

**AEC-sensitivity analysis.** *T. thermophilus* cells were pre-cultured in TM medium at 70 °C overnight. The cells were washed 2 times with MM media and suspended in an equal volume of MM medium. Then, 0.1% of the culture was transferred into fresh MM
medium supplemented with or without 500 μM AEC. The strains were grown aerobically at 70 °C and O.D.₆₀₀ of the cultures was monitored at every 3 h.

**Pull-down assay.** TTC0795 with Strep-tag at the N-terminus, TTC0806 with His₆-tag at the N-terminus, and TTC0807 with FLAG-tag at the C-terminus were separately produced in *E. coli* BL21 RIL-Codon Plus (DE3) cells. All PCR primers used for the pull-down assay are listed in Supplemental Table S3. Harvested cells were suspended in 32 ml buffer II, washed, and disrupted by sonication. The supernatant was prepared by centrifugation at 40,000 x g for 20 min. The precipitate containing TTC0806 was suspended in 20 ml buffer IV (20 mM Tris-HCl, pH8.0, 300 mM NaCl, 20% glycerol, 0.8% dodecyl maltoside), agitated for solubilization at 4 °C for 2 h, and centrifuged at 40,000 x g for 30 min. TTC0795 was purified with a Strep-Tactin column (Novagen) according to the manufacturer’s instructions. The supernatant containing TTC0806 was applied to an Ni²⁺-NTA column pre-equilibrated with buffer II supplemented with 20 mM imidazole and 0.03% dodecyl maltoside. After washing with buffer II containing 100 mM imidazole and 0.03% dodecyl maltoside, adsorbed proteins were eluted with buffer II supplemented with 500 mM imidazole and 0.03% dodecyl maltoside. TTC0807 with FLAG tag was purified as follows. The supernatant containing TTC0807 was applied to an ANTI-FLAG M2 affinity column (Sigma Aldrich) pre-equilibrated with buffer V (50 mM Tris-HCl, pH 7.4, 150 mM NaCl). After washing with the same buffer, adsorbed TTC0807 was eluted with buffer V supplemented with FLAG peptide (Sigma Aldrich) at 0.1 mg ml⁻¹. Purified TTC0795, TTC0806, and TTC0807 were dialyzed with buffer II, buffer II supplemented with 0.03% dodecyl maltoside, and buffer V, respectively. TTC0806 (His₆-tag) was mixed with either TTC0795 (Strep-tag) or TTC0807 (FLAG-tag), and incubated for 1 h at room temperature. TTC0806 (His₆-tag) mixed with TTC0795 (Strep-tag) was applied to an Ni²⁺-NTA column, and adsorbed proteins were eluted with buffer II supplemented with 500 mM imidazole and 0.03% dodecyl maltoside.
A similar experiment was performed for a mixture from which TTC0806 (His\(_8\)-tag) was omitted. TTC0806 (His\(_8\)-tag) mixed with TTC0807 (FLAG-tag) was applied to an ANTI-FLAG M2 affinity column, and adsorbed protein was eluted in the same way as above using FLAG peptide at 0.1 mg ml\(^{-1}\). A similar experiment was performed for a mixture from which TTC0807 (FLAG-tag) was omitted. Both eluates were separated on 12% SDS-PAGE.

**Preparation of TTC0807 for crystallization.** Because TTC0807 was predicted to encode a periplasmic substrate-binding protein with a signal peptide (Met1 to Ala18) for periplasmic localization by SignalP 4.0 server (16), a DNA fragment corresponding to the mature protein (Gln19 to Lys254) was amplified by PCR with a pair of synthetic oligonucleotides listed in Supplemental Table S4, digested with EcoRI/HindIII, and cloned into pBluescript II KS (+). A DNA fragment with the desired sequence was cloned into the EcoRI/HindIII site of pHIS8 (17) to yield pHIS8-0807 and introduced into *E. coli* BL21-CodonPlus (DE3)-RIL cells. Using this construct, TTC0807 was designed to be produced in a form with His\(_8\)-tag at the N-terminus. *E. coli* cells harboring pHIS8-0807 were cultured in 2 x YT medium supplemented with 50 \(\mu\)g/ml kanamycin and 30 \(\mu\)g/ml chloramphenicol at 37 °C. When the *E. coli* cells were grown to O.D.\(_{600}\) of 0.6, IPTG was added at a final concentration of 0.1 mM. Culture was continued at 25 °C for an additional 12 h. TTC0807 was purified as follows. *E. coli* cells harboring pHIS8-0807 from 1.6 liter culture were harvested, washed, and suspended in 32 ml buffer II. Suspended cells were disrupted by sonication. The supernatant prepared by centrifugation at 40,000 x g for 20 min was heated at 70 °C for 30 min, and denatured proteins from *E. coli* cells were removed by centrifugation at 40,000 x g for 20 min. Supernatant was applied to an Ni\(^{2+}\)-NTA resin column which was pre-equilibrated with buffer II supplemented with 20 mM imidazole. After washing the column with a sufficient volume of the same buffer, TTC0807 was eluted with buffer II supplemented with 500 mM imidazole. The eluted
fraction was concentrated with a Vivaspin concentrator MWCO 10,000 Da (Sartorius Stedim Biotech., NY) and then applied to a Superdex 200 pg HiLoad 26/60 gel filtration chromatography column (GE Healthcare) equilibrated with buffer II. The TTC0807-containing fractions were concentrated with a Vivaspin concentrator MWCO 10,000 Da. The purity of the recombinant protein was verified by 12% SDS-PAGE. The protein concentration was adjusted to 10 mg ml\(^{-1}\) and used for crystallization.

**Crystallization of TTC0807.** Crystallization conditions of TTC0807 with AEC, lysine, ornithine, and arginine were screened by the hanging drop vapor diffusion method using screening kits commercially available. Drops of 2 \(\mu\)l consisting of reservoir solution (1 \(\mu\)l) and 10 mg/ml TTC0807 solution (1 \(\mu\)l) supplemented with 10 mM lysine, AEC, ornithine or arginine were equilibrated against 500 \(\mu\)l reservoir solution at 293 K. Crystals of TTC0807 with AEC, lysine, ornithine, and arginine were obtained from droplets using a solution (0.2 M ammonium sulfate and 20 % PEG 4000). Crystals under each condition were grown in a few days.

**Structure determination of TTC0807 complexed with AEC, lysine, ornithine, and arginine.** Prior to data collection, crystals were briefly soaked in reservoir solution supplemented with 20 % (v/v) PEG 400 as a cryoprotectant, flash-cooled in a nitrogen gas stream at 95K, and stored in liquid nitrogen. Diffraction data of TTC0807 with lysine, AEC and ornithine were collected at the NW12 station of the Photon Factory, High Energy Accelerator Research Organization (KEK) (Tsukuba, Japan). Diffraction data of TTC0807 with arginine were collected at the NE-3A station of the Photon Factory. Diffraction images were indexed, integrated, and scaled using the HKL-2000 program suite (18). Details of data collection statistics are summarized in Table 1. The data of TTC0807 crystals collected at a wavelength of 1.0 Å were used for subsequent molecular replacement and crystallographic refinement. Molecular replacement was performed.
with Phaser (19) in the CCP4 program suite (20) using the model of LAO-binding protein
(StLAO-BP) from *Salmonella typhimurium* (PDB code, 1LST) (21). Refinement was
performed with Refmac 5.5 (22) and model correction in the electron density map was
performed with Coot (23). Data collection, refinement statistics, and the results of
Ramachandran plots produced by the program PROCHECK (24) are summarized in
Table 1. The figures were prepared using PyMOL (available on the World Wide Web).

Crystallographic data accession number. The atomic coordinates and structure
factors for TTC0807/AEC, TTC0807/ornithine, TTC0807/lysine, and TTC0807/arginine
complexes have been deposited in the Protein Data Bank (PDB codes 3VV5, 3VVD,
3VVE, and 3VVF).
RESULTS

Isolation of AEC-resistant mutants. To elucidate the mechanism of hypersensitivity to AEC of *T. thermophilus* HB27, we isolated mutants that exhibited AEC resistance by NTG mutagenesis. The mutagenized cells were spread on an MM medium plate supplemented with 500 μM AEC. Although the wild-type strain showed no growth under this condition (Fig. 2), eight colonies could grow on the plate containing AEC. From these eight mutants, two strains named AT10 and AT14 (*AEC*-resistant *T. thermophilus No. 10* and No. 14) were chosen for further analyses.

Identification of mutations responsible for AEC resistance.

a) AT14

Since *T. thermophilus* exhibits natural competence, DNA can be easily taken up into the cells and integrated into the genome by double-crossover homologous recombination. We obtained approximately 200 cosmids carrying DNA from AT14 mutant and screened for the DNA fragment that conferred AEC resistance on the wild-type strain. We isolated two cosmids, cos14-204 with a 23 kb insert and cos14-277 with a 33 kb insert, which gave AEC resistance to *T. thermophilus*, from AT14. Both the cosmids carried the same DNA region from TTC0795 to TTC0810, suggesting that this region should contain (a) mutations responsible for AEC resistance of AT14. Sequencing of the region revealed that there were two in-frame deletions of 3 bp, causing a loss of Gln143 of TTC0795 and a loss of Leu175 of TTC0969 on the AT14 genome (Fig. 3A).

The above result suggests that loss of function of either of these genes or both is a possible cause of the AEC resistance of AT14. Interestingly, each of TTC0795 and TTC0969 is a component of ABC transporters. ABC transporters are composed of two transmembrane domains and two cytoplasmic nucleotide-binding domains that hydrolyze ATP to drive transport. In Gram-negative bacteria, ABC transporters require a binding
protein that binds the substrates to be imported and transfers them to the transporters. TTC0969 is annotated as a gene encoding nucleotide-binding subunit (NBD) of ABC transporters. TTC0969 forms a cluster with flanking four genes, TTC0966, TTC0967, TTC0968, and TTC0970 (Fig. 3D). TTC0966 is annotated to encode a periplasmic substrate-binding protein for ABC transporters. Both TTC0967 and TTC0968 are annotated to code for membrane subunits (MPs) of ABC transporters, while TTC0970 is annotated to encode the NBD of ABC transporters, suggesting that these five proteins are involved in solute uptake. On the other hand, TTC0975 is also annotated as a gene encoding an NBD component of ABC transporters. As described below, TTC0975 is suggested to be an NBD component that interacts with TTC0806, a transmembrane subunit, of ABC transporters. TTC0806 forms a gene cluster with TTC0807 (Fig. 3E), which is annotated as a gene encoding periplasmic amino acid-binding protein for ABC transporters.

These two missing residues are located away from the substrate-binding residues on the respective proteins based on the crystal structures of PDBID: 2OLJ (NBD of arginine transporters from *Geobacillus stearothermophilus* for TTC0975 and PDBID: 1G6H (NBD of ABC transporter from *Methanocaldococcus jannaschii* for TTC0969 (25); therefore, we hypothesized that loss of the amino acid residues might affect protein stability. In order to examine this possibility, TTC0975-ΔGln143 was heated at elevated temperatures and undenatured fractions were subjected to SDS-PAGE (Fig. 3AB). Analysis revealed that the mutant was completely inactivated at 50.2 °C, which was considerably lower than 63.5 °C for the wild-type TTC0975. Similar analysis was performed for TTC0969-ΔLeu175. The mutant was also inactivated at a lower temperature than wild-type TTC0969. These observations indicate that two proteins involved in ABC transporter function are heat-labile in AT14. In accordance with this, AT14 exhibited AEC sensitivity in culture at 50 °C, which contrasts with the culture at 70 °C (Fig. 3C).
Similar experiments were conducted for the AT10 cosmid library. Unexpectedly, the simultaneous introduction of two cosmids, cos10-3 and cos10-5, was required to confer AEC resistance on *T. thermophilus* HB27. Sequence analyses of the inserts on cos10-3 (38 kb) and cos10-5 (34 kb) revealed that there was an inversion of a locus between *TTC0807* and *TTC0969* on the AT10 genome, which explains the necessity of simultaneous introduction of the two cosmids to cause inversion of the wild-type genome.

Around the junction points of *TTC0807* and *TTC0969*, similar nucleotide sequences were found, and this might assist genome inversion between these loci (Fig. 4ABC). At the junction points of the AT10 genome, two TTGAT sequences were inverted.

**Deletion of genes for proposed AEC transporters.** To confirm further the involvement of the mutations in AEC resistance, we constructed single and double mutants of *TTC0807* and *TTC0969*, and examined the growth of the *T. thermophilus* mutants lacking these genes. When single-site mutants, Δ*TTC0807* and Δ*TTC0969*, were cultured in MM medium containing 500 μM AEC, these mutants grew very slowly, which contrasts with the double mutant, Δ*TTC0807/TTC0969*, which was capable of growing rapidly with a growth rate similar to that of AT10 (Fig. 5). These results indicate that two different transporters, in each of which either TTC0807 or TTC0969 is involved, contribute to AEC uptake in *T. thermophilus* HB27. We hereafter call the two ABC transporters AecT-I and AecT-II: TTC0806 and TTC0807 are involved in AecT-I function and TTC0967-TTC0970 are involved in AecT-II function. In addition to *TTC0807* and *TTC0969*, we also constructed mutants lacking either *TTC0795* or *TTC0806* (Supplementary Fig. S1). Δ*TTC0806* strain grew with a profile similar to that of Δ*TTC0807*, suggesting that these products are used as mechanisms in the same AEC uptake system. The growth profile of Δ*TTC0795* mutant is also similar to those of
ΔTTC0806 and ΔTTC0807, suggesting that TTC0795 is the subunit of ABC transporters working with TTC0806.

Interaction of TTC0806 with TTC0795 and TTC0807. As described above, mutations in both AT10 and AT14 resulted in malfunctioning of two AEC uptake systems. AecT-II is composed of TTC0967-TTC0970 using TTC0966 as a periplasmic substrate-binding protein. The other transporter AecT-I contains TTC0806 as an MP using TTC0807 as a periplasmic substrate-binding protein. ΔTTC0795 mutant responded to AEC similarly to ΔTTC0806 and ΔTTC0807 mutants. Therefore, we hypothesized that TTC0795 functions as a component of AecT-I with TTC0806 using TTC0807 as a periplasmic substrate-binding subunit. To examine this hypothesis, a pull-down assay was performed using the three components with different tags. TTC0806 with N-terminal His-tag, TTC0807 with C-terminal FLAG-tag, and TTC0795 with N-terminal Strep-tag were separately produced in E. coli cells, and purified as described in Materials & Methods. When TTC0806 (His8-tag) was mixed with TTC0795 (Strep-tag) and applied to Ni²⁺-NTA column, TTC0795 (Strep-tag) was co-eluted with TTC0806 (His8-tag) by addition of 500 mM imidazole (Fig. 6A). Since TTC0795 (Strep-tag) alone was not adsorbed to Ni²⁺-NTA column (Fig. 6A), this result indicates that TTC0795 can interact with TTC0806. This supports the above-mentioned assumption that TTC0795 is an NBD component of AecT-I to take up AEC. When TTC0806 (His8-tag) was mixed with TTC0807 (FLAG-tag) and applied to ANTI-FLAG M2 column, unexpectedly addition of FLAG peptide caused elution of only TTC0807 (FLAG-tag) (Fig. 6B).

Crystal structure of TTC0807 complexed with amino acids. TTC0807, which is a periplasmic substrate-binding protein for AecT-I, was overexpressed, purified, and crystallized with AEC, lysine, ornithine, and arginine. Crystal structures of TTC0807 complexed with AEC, lysine, ornithine, and arginine were determined at 1.90, 2.00, 2.07,
and 1.90 Å, respectively. DALI search (26) showed that the structure of TTC0807 was most similar to that of LAO-binding protein, StLAO-BP, from *Salmonella typhimurium* with a Z-score of 30.6 and root mean square deviations of 1.3 Å for Cα atoms (Fig. 7AB). StLAO-BP is composed of domain 1 (A1-Y88 and T195-K238) and domain 2 (R93-V185) connected by the domain-connecting regions (DCR1: A89-S92 and DCR2: K186-G194) (Fig. 7C). Although the structure of TTC0807 is principally similar to that of StLAO-BP, DCRs exhibit different structures. StLAO-BP, DCR1 is flexible with a random coil structure and DCR2 is mostly in a random coil containing a short α-helix in the middle. In contrast, in the TTC0807 structure, β8 (DCR1) and β12 (DCR2) forming a β sheet connect the two domains. Cys97 in the DCR1 forms a disulfide bond with Cys235 in TTC0807. The disulfide bond is likely a structural factor that enhances the thermostability of domain 1. Lysine and other substrates are bound in the pocket between these two domains (Figs. 7A & 8A-E). The asymmetric unit contains two TTC0807 molecules. Although AEC is bound to B-chain in a single conformation, it is bound to A-chain in alternative conformations (Fig. 8AB). In the B-chain, the side-chain amino group of AEC is recognized only by Glu19 side-chains, whereas in A-chain the side-chain amino group of AEC is found at two different positions. In both conformations the amino group forms electrostatic interactions with Glu19; however, in one form it forms a hydrogen bond with Gln123 and in the other form it is hydrogen-bonded with Asn26. Other portions of AEC molecule are recognized in the same way. In both chains, the α-amino group of AEC interacts with Glu191 by electrostatic interaction and further forms hydrogen bonds with the side-chain hydroxyl group of Tyr128 and the main-chain carboxyl group of Ser78, and the α-carboxyl group forms electrostatic interactions with Arg85. The α-carboxyl group is also recognized by hydrogen bonds with the main-chain amide group of Gly80 and the side-chain hydroxyl group of Thr127 (Fig. 8A). The side-chain hydrophobic portion of AEC was stacked with two aromatic residues Phe22 and Phe60. In arginine, ornithine, and lysine-bound complexes, two chains in the
asymmetric unit of those crystal structures bind the substrates in a single conformation. In these complexes, the substrates are bound in a similar manner using the above-mentioned residues (Fig. 8A-E). Interestingly, in order to bind lysine and ornithine, the side chain of Glu19 was rotated 33° to form suitable electrostatic interactions with the substrates. In the TTC0807/arginine complex, arginine is bound to the enzyme with the side chain of Glu19 in a conformation similar to that for binding AEC. The position of Glu19 is necessary to accommodate the larger guanidium group of arginine in the binding pocket (Fig. 8C). It is of interest that the conformation of Glu19 in the AEC complex is very similar to that in the arginine complex but not to that in the lysine complex, although AEC is an analog of lysine. This is because AEC has a sulfur atom in the γ-position. Since C-S bond length (1.8 Å) is longer than that of C-C bond (1.5 Å), AEC is larger than lysine maximally by 0.6 Å, although it is smaller than that of arginine. Regardless, TTC0807 recognizes these basic amino acids by changing the conformation of the Glu19 side chain.

**Comparison of lysine recognition with other lysine-binding proteins.** When the TTC0807/lysine complex is compared with the lysine-bound complexes of StLAO-BP and ArtJ from *Geobacillus stearothermophilus* (GsArtJ) (27, 28), lysine is recognized in a slightly different manner, although Arg85 (in TTC0807 numbering), which interacts with the α-carboxyl group, is conserved in StLAO-BP and GsArtJ, and similar recognition of the α-amino group of lysine is observed with the main chain carbonyl oxygen of Ser78 and the corresponding residues in the three proteins (Figs. 7C & 8EFG). In TTC0807, the α-amino group of lysine is recognized by Tyr128 and Glu191 (Fig. 8E), whereas it is recognized by Ser70 and Asp161 in StLAO-BP and Gly86, Thr88, and Asp175 in GsArtJ (Fig. 8FG). Interestingly, although Asp161 in StLAO-BP (Asp175 in GsArtJ) is conserved as Asp165 in TTC0807, Asp165 does not interact with the α-amino group of lysine. Instead, Tyr128 recognizes the α-amino group in TTC0807. Tyr128 is replaced with Gln in StLAO-BP and GsArtJ. The α-amino group of lysine is also recognized by
Glu191 from β12 in TTC0807. In StLAO-BP and GsArtJ, the corresponding residue is conserved as Asp193 and Glu203 with a similar negative charge, respectively; however, these residues do not participate in substrate binding. Thus, recognition of the α-amino group of lysine is different between TTC0807 and the other two proteins.

The ε-amino group of lysine is also recognized in a different way among these three lysine-binding proteins. The amino group is recognized by Gln123 and Glu19 in TTC0807, while it is recognized by Asp11 and a bound water molecule in StLAO-BP in which the residue corresponding to Gln123 is replaced with Leu117 (Supplementary Fig. S3). In GsArtJ, Gln132 occupies a similar position to Gln123 in TTC0807; however, it is different in that the bound water molecule directly interacts with the ε-amino group of lysine. Taken together with the α-amino group recognition, lysine is recognized using similar but distinct mechanisms by these related proteins.

In the AEC-bound complex of TTC0807, the side-chain amino group of AEC is recognized by Glu19 and either Asn26 or Gln123. Asn26 and Gln123 in TTC0807 are replaced with Ser18 and Leu117 in StLAO-BP, which cannot form a hydrogen bond with the amino group, suggesting weak binding of the amino group in StLAO-BP. In addition to the difference in α-amino group recognition, recognition of the side-chain amino group is stabilized by tight binding in TTC0807, suggesting that the AEC molecule is bound by TTC0807 more tightly than by StLAO-BP and possibly the ortholog in E. coli; therefore, we speculate that tight binding of AEC in TTC0807 is one of the factors responsible for the hypersensitivity of T. thermophilus to AEC.

**DISCUSSION**

AEC is a lysine analog that inhibits the growth of bacterial cells by reduction of AK activity in cells, resulting in decreased flow toward lysine biosynthesis, and/or by mis-incorporation into protein. Compared to E. coli and C. glutamicum, T. thermophilus
exhibits higher sensitivity to AEC. In this study, we isolated AEC-resistant mutants of *T. thermophilus* to elucidate the mechanism of the hypersensitivity to AEC of *T. thermophilus* by identification of the mutated genes.

When two AEC-resistant mutants, AT10 and AT14, were analyzed, they were found to carry mutations that resulted in malfunctioning of two different solute-uptake systems. Both mutants were isolated by NTG treatment; however, NTG treatment may not be the direct cause of mutations occurring on the genomes of the mutants, because the mutagen is known to cause G:C to A:T transition. In AT10, genome inversion was found, while 3 bp in-frame deletions were found in two genes in AT14. A genome rearrangement with insertion of an inverted repeat sequence in AT10 is reminiscent of an IS element-mediated genome arrangement. Regardless, this inversion was not expected by NTG mutagenesis. At present, the mechanisms of this genome inversion and the in-frame deletions remain unknown.

Genes encoding components and their cognate solute-binding protein of AecT-II are clustered on the genome as *TTC0966-TTC0970*; however, those for AecT-I are located in two regions. Among them, *TTC0806* and *TTC0807* may be transcribed with *TTC0805* encoding putative valyl-tRNA synthetase in *T. thermophilus* (Supplementary Fig. S2). In the genome of the related microorganism, *Thermus scotoductus* SA-01 (29), the DNA region corresponding to *TTC0793-TTC0805* in *T. thermophilus* HB27 is present in the opposite direction, and ORF corresponding to *TTC0795* is located downstream of *TTC0806* intervened by two ORFs encoding a putative TRAP transporter and its cognate periplasmic substrate-binding protein (Supplementary Fig. S2). In another related microorganism, *Marinithermus hydrothermalis* (30), three ORFs encoding MP, NBD, and substrate-binding protein, which are absent in *T. thermophilus*, intervened between ORFs corresponding to *TTC0795* and *TTC0806* (Supplementary Fig. S2). We speculate that gene inversion occurred in *T. thermophilus* HB27 after separation of *T. thermophilus* from the ancestral species. ABC transporters are composed of two MPs and two NBDs.
We assume that AecT-I comprises two TTC0795 subunits and two TTC0806 subunits using TTC0807 as the periplasmic substrate-binding protein; however, there is still a possibility that other unidentified MP and NBD species are co-components of AecT-I with TTC0795 and TTC0806.

TTC0806 formed a gene cluster with TTC0807, and ∆TTC0806 strain grew with a profile similar to that of ∆TTC0807. These observations suggest that TTC0806 and TTC0807 are used as mechanisms in the same AEC uptake system. In the pull-down assay, although interactions between TTC0806 and TTC0795 were observed, interactions between TTC0806 with TTC0807 were not detected. We assume that the periplasmic region of TTC0806, a membrane protein, cannot form an appropriate structure that can be recognized by TTC0807 without a membrane.

Single-site mutants, ∆TTC0807 and ∆TTC0969, were able to grow in MM medium containing 500 μM AEC; however, these mutants grew very slowly, which contrasts with the double mutant, ∆TTC0807/TTC0969, which was capable of growing rapidly with a growth rate similar to that of AT10. These observations suggest that both transporting systems are involved in AEC uptake. When the growth profile was compared between ∆TTC0807 and ∆TTC0969 strains, ∆TTC0807 strains entered the logarithmic phase before 40 h cultivation, whereas ∆TTC0969 strain required a longer incubation time to enter the logarithmic phase. Considering that ∆TTC0807 and ∆TTC0969 strains still possess native AecT-II and AecT-I systems, respectively, to take up AEC, we assume that AecT-I possesses stronger ability than AecT-II to import AEC into the cytoplasm.

It is known that substrate binding causes distinct rigid body motion to close the substrate-binding pocket by bond rotation in DCR regions in periplasmic substrate-binding protein (21). Since crystals of TTC0807 have not yet been obtained in an apo form, we cannot infer whether such a marked structural change is induced upon substrate binding in TTC0807 at present. In other periplasmic substrate-binding proteins such as StLAO-BP and GsArtJ, DCR2 forms an α-helix (α8 in StLAO-BP) that is flanked...
by β strands, β11 and β12 (Fig. 7BC). In contrast, in TTC0807, DCR2 takes the form of a β strand to form a long and stable β-sheet with β8 from DCR1 in every substrate-binding complex. Since Glu191 from DCR2 forms a stable salt bridge with the α-amino group of the substrates, we assume that substrate binding will induce domain closure, triggered by the displacement of Glu191, which will be elucidated experimentally in the near future.

In this study, we identified two ABC transporter-mediated AEC uptake systems in T. thermophilus and determined the crystal structures of TTC0807, a periplasmic basic amino acid-binding protein, for AecT-I. We think that these AEC uptake systems may contribute to the hypersensitivity of T. thermophilus to AEC. However, we also think that it is necessary to identify a cytoplasmic molecular target of AEC other than HCS to clarify the hypersensitivity.

ACKNOWLEDGMENTS

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REFERENCES


FIGURE LEGENDS

Fig. 1. Chemical structures of lysine and AEC.

Fig. 2. Growth profiles of AT10 and AT14 in MM medium containing 500 μM AEC. Open square, AT14; closed square, AT10; open diamond, wild type.

Fig. 3. Mutation sites in AT14. (A) Deletion of Gln143 in TTC0975 (upper panel) and comparison of heat stability between TTC0975 proteins from wild-type strain and AT14 (lower panel). Lanes 1, not heated; 2, 31.0°C; 3, 34.1°C; 4, 36.1°C; 5, 39.0°C; 6, 41.0°C; 7, 42.4°C; 8, 45.2°C; 9, 50.2°C; 10, 56.0°C; 11, 60.8°C; 12, 63.5°C; 13, 65°C. (B) Deletion of Leu175 in TTC0969 (upper panel) and comparison of heat stability between TTC0969 proteins from wild-type strain and AT14 (lower panel). Lanes are the same as in (A). (C) Growth of AT14 in MM medium at permissive and non-permissive temperatures. Lanes 1 and 2, cultured at 70°C; lanes 3 and 4, cultured at 50°C. Lanes 1 and 3, in the absence of 500 μM AEC; lanes 2 and 4, in the presence of 500 μM AEC. (D) TTC0966-TTC0970 region in wild-type T. thermophilus HB27. (E) TTC0793-TTC0807 region in wild-type T. thermophilus HB27. Periplasmic substrate-binding protein is abbreviated as SBP.

Fig. 4. Two cosmids from AT10 necessary for conferring AEC resistance to wild-type strain. (A) Inversion of a locus between TTC0807 and TTC0969 on the AT10 genome and schematic structures of inserts in cos10-3 and cos10-5. Only a few open reading frames are shown but portions containing other open reading frames are shown as broken lines. (B) Nucleotide sequences around the recombination sites in TTC0807 and TTC0969. (C) Sequence comparison between the recombination sites.

Fig. 5. Effect of deletion of TTC0807 and/or TTC0969 on growth in MM medium containing...
500 μM AEC. Closed square, AT10; open diamond, wild type; open circle, ΔTTC0807 mutant; closed circle, ΔTTC0969 mutant; closed triangle, ΔTTC0807/ΔTTC0969 double mutant.

Fig. 6. Pull-down assay for TTC0806, TTC0807, and TTC0795. (A) pull-down assay for TTC0795 and TTC0806. Lane 1 Molecular weight marker; lane 2, eluate from Ni\(^{2+}\)-NTA column for only TTC0795 (Strep-tag); lane 3, eluate from Ni\(^{2+}\)-NTA column for mixture of TTC0795 (Strep-tag) and TTC0806 (His\(_8\)-tag); lane 4, purified TTC0795 (Strep-tag); lane 5, purified TTC0806 (His\(_8\)-tag). (B) pull-down assay for TTC0806 and TTC0807. Lane 1 Molecular weight marker; lane 2, eluate from ANTI-FLAG M2 affinity column for only TTC0806 (His\(_8\)-tag); lane 3, eluate from ANTI-FLAG M2 affinity for mixture of TTC0806 (His\(_8\)-tag) and TTC0807 (FLAG-tag); lane 4, purified TTC0806 (His\(_8\)-tag); lane 5, purified TTC0807 (FLAG-tag).

Fig. 7. Structure of TTC0807. (A) Crystal structure of TTC0807/lysine complex. (B) Crystal structure of StLAO-BP/lysine complex. Domain-connecting regions (DCRs) in StLAO-BP and the corresponding portions in TTC0807 are colored magenta. N and C represent N- and C-terminal domains, respectively. Disulfide bond in TTC0807 is shown as S-S bond. (C) Amino acid sequence alignment of TTC0807 and homologs. Secondary structures are also shown. Amino acid residues involved in substrate binding and in disulfide-bond formation are marked by blue and yellow circles, respectively. DCRs are shown by magenta boxes and putative loop necessary for interaction with a transmembrane component of ABC transporters is indicated by a brown box.

Fig. 8. Ligand-binding site. (A) A-chain of TTC0807/AEC complex. (B) B-chain of TTC0807/AEC complex. (C) TTC0807/arginine complex. (D) TTC0807/ornithine complex. (E) TTC0807/lysine complex. (F) StLAO-BP/lysine complex. (G) GsArtJ/lysine complex.
Nitrogen, oxygen, and sulfur atoms are shown as blue, red, and orange sticks, respectively.

Carbon atoms of amino acid residues of TTC0807, StLAO-BP, and GsArtJ are shown as green, cyan, and yellow sticks, respectively. Carbon atoms of bound molecules of AEC, arginine, ornithine, and lysine are shown as light magenta, brown, yellow, and dark magenta sticks, respectively. The electron density maps (2Fo-Fc map) of AEC in A-chain, AEC in B-chain, arginine, ornithine, and lysine are shown as meshes contoured at 1.3, 1.3, 1.5, 1.6, and 1.6 \( \sigma \), respectively.
Table 1. X-ray data collection and refinement statistics

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aValues in parentheses are for the highest resolution shell.

b$R_{merge} = \Sigma|I_i - <I>|/\Sigma<I>$.

Lysine, ornithine, and arginine are abbreviated as Lys, Orn, and Arg, respectively.
Lysine 2-Aminoethyl-cysteine

Kanemaru, Y., Hasebe, F., et al., Figure 1.
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Kanemaru, Y., Hasebe, F., et al., Figure 7.
Kanemaru, Y., Hasebe, F., et al., Figure 8.